Biological Control of Crown Gall with Fungal and Bacterial Antagonists

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ABSTRACT

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Pathogenic *Agrobacterium* strains were inhibited in vitro by 35 different fungi and bacteria that were isolated from nursery soils in Oregon and Washington. Seven of the 35 antagonists inhibited six *A. tumefaciens* strains in vitro, and also prevented infection of tomato seedlings in the greenhouse. In field tests, isolates of *Penicillium, Aspergillus, Bacillus, Pseudomonas,* and *A. radiobacter* reduced the incidence of galling on mazzard cherry seedlings by some of the pathogens tested, but none was more effective than *A. radiobacter* strain 84. With some antagonists, greater reduction in galling was observed when cherry seedlings were inoculated with pathogen mixtures 24 hr after inoculation with antagonists. One *Penicillium* antagonist reduced the incidence of infection to a level below that of the wounded controls when used against some pathogenic strains.

Successful biological control of crown gall with strain 84 of *Agrobacterium radiobacter* has been reported in several countries, and has been reviewed recently (8,14,15). However, some strains of *A. tumefaciens* were insensitive to the bacteriocin (agrocin 84) produced by strain 84 in vitro (9,10,14,17), and in some instances strain 84 did not prevent tumor production by these pathogens on susceptible hosts (10,14). The success of strain 84 has encouraged workers to look for new antagonists for the strain 84-insensitive pathogens, but other *A. radiobacter* strains that inhibit pathogenic *Agrobacterium* species in vitro have been ineffective as control agents on plants (5,10,13).

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Soil fungi are a potential source of antagonists that have been largely ignored in crown gall research. Deep and Young (3) showed that preplanting fungicide treatments increased the incidence of crown gall of cherry seedlings, suggesting the presence of natural fungal competitors. They later used a suspension of unidentified fungi as a preplanting treatment and reduced galling by about 25%. The purpose of the present study was to isolate fungal and bacterial antagonists for biological control of crown gall, particularly for control of *A. tumefaciens* strains not subject to control by strain 84.

MATERIALS AND METHODS

Isolation of antagonists. Soil samples from nursery fields in Oregon and Washington were collected as composite samples

consisting of six 15-cm-long core samples (2 cm in diameter) from each field. The soil samples were mixed thoroughly and screened for microorganisms antagonistic toward pathogenic *Agrobacterium* strains by a plate-spraying technique similar to that of Stessel et al (18). Soil dilutions were plated on Difco potato-dextrose agar (PDA), allowed to incubate at room temperature for 1–3 days, and then were sprayed with standardized cell suspensions of either strain B6 of *A. tumefaciens* (a biotype 1 strain insensitive to the biological control agent, *A. radiobacter* 84) or strain B234 of *A. tumefaciens* (biotype 2, sensitive to strain 84). Inoculum for these two test strains was prepared from late log-phase cultures grown in a yeast-dextrose-peptone broth (13) on a rotary shaker. Test strain cultures were diluted to about 1×10^8 cells per milliliter with sterile distilled water, and sprayed onto plates until evenly wetted, but not to runoff. Microbial colonies exhibiting zones of inhibition against the test strains were isolated from the agar and obtained in pure culture.

Antiobiotic production in vitro. Each antagonist selected in the initial screening was retested in vitro against strains B6 and B234, and also was tested against an additional biotype 1 pathogen that was agrocin 84-insensitive (EU-8) and three additional biotype 2 pathogens that were agrocin 84-sensitive (Q51, K27, and K29). A modification of the method of Pridham et al (16) was used for antagonists other than the *A. radiobacter* strains. Plugs (7-mm diameter) cut from agar plate cultures of the antagonists were placed on PDA plates that had just been sprayed with the test strain, thus avoiding spread of nonchloroformed antagonists over the agar surface. This method provided clear zones of inhibition against the pathogens by all antagonists except the agrobacteria. A method similar to Stonier's (19) was used for *A. radiobacter* strains which were spotted at the center of mannitol-glutamate (MG) agar (6) plates, and then grown for 3 days at room temperature before being sprayed with the test strains. The antagonists were not killed with chloroform after the initial incubation period as described by Stonier, since little or no spread of the colonies resulted from spraying the test strains. Zones of inhibition appeared after 1–2 days in lawns of sensitive strains of *A. tumefaciens*. Antagonists that inhibited all six pathogenic test strains, or strains that were resistant to strain 84, were screened for control of galling of tomato seedlings in the greenhouse.

Greenhouse experiments. Assays for the prevention of galling were made by wounding 4-wk-old tomato seedlings (*Lycopersicum esculentum* 'Bonny Best') in the first internode with a dissecting needle, forming a slit 2–3 mm long and 1.0–1.5 mm deep in the stem, and placing 0.01 ml of inoculum on the wound. Inoculum was prepared by washing cells from PDA or MG agar slants with sterile distilled water, and diluting to the desired concentration. Inocula of the pathogen and antagonist were mixed in a 1:10 ratio and applied to the wounds. Seedlings were then grown for 4 wk before final readings were made. On the basis of these tomato assays seven antagonists, which included species of *Penicillium, Aspergillus, Bacillus, Pseudomonas,* and *Agrobacterium,* were selected for field trials.

Further tomato assays were conducted with the seven antagonists against mixtures of pathogenic strains which also were used in field trials. Mixtures of pathogenic strains were used because natural populations of *A. tumefaciens* can contain more than a single strain. Evidence for a heterogeneous pathogen population comes from the observation that agrobacteria of different biotypes, and with different host specificities, can be isolated from a single tumor (1). In the present study, two groups of pathogens were used. Group I consisted of four pathogenic strains (A49, A432, A20, and A329) that were insensitive to strain 84 in vitro. Group II consisted of two 84-insensitive strains (B6 and EU-8) and two 84-sensitive strains (B234 and U-3). Pathogen-antagonist mixtures were inoculated directly to stem wounds, as described for the tomato assays, or the pathogens were applied 24 hr after the antagonists.

Field tests for biological control. Before being planted, mazzard cherry seedlings were root-pruned and dipped in inoculum suspensions of individual antagonists, then immediately dipped in suspensions of the pathogens. This sequence of inoculation gave effective control of crown gall with *A. radiobacter* strain 84 in previous work (3). Alternatively, seedlings were inoculated with antagonists, held for 24 hr in a cool moist condition, and inoculated with pathogens just before planting. The 24-hr delay was used because root-pruned, inoculated plants are sometimes held for extended periods before planting if excessive rain occurs, and because delay between inoculation with *A. radiobacter* 84 and pathogens enhances control (13). Mixtures of *A. tumefaciens* were prepared in nonchlorinated tap water in the field from strains grown separately on MG medium. Viability counts of the inocula were made within 24 hr of use by plating serial dilutions on PDA.

RESULTS

Antibiotic production in vitro. In the initial screening from soil, 35 different fungi and bacteria inhibited the test strains B6 or B234 of *A. tumefaciens*. Twelve of these antagonists inhibited B6, B234, and an additional four pathogenic test strains (Q51, K27, K29, and EU-8). Four antagonists were effective against only the biotype 1 pathogens, B6 and EU-8 (Table 1). The remaining 19 antagonists were effective against only one or two biotype 2 strains, and thus were not included in greenhouse tests.

TABLE 1. In vitro sensitivity of *Agrobacterium tumefaciens* to bacterial and fungal antagonists

Antagonists	Diameter of inhibition zone (cm) against various *A. tumefaciens* strains[a]					
	B6	EU-8	B234	Q51	K27	K29
Aspergillus 77-148	3.5	2.2	2.0	0.0	0.0	1.6
Aspergillus 77-149	1.5	1.1	0.0	0.0	0.0	0.0
Penicillium 77-15	1.5	1.1	0.0	0.0	0.0	0.0
Penicillium 77-104	3.1	2.6	5.5	3.7	4.6	4.0
Penicillium 77-166	1.0	3.0	0.0	0.0	0.0	0.0
Penicillium 77-170	1.2	3.0	0.0	0.0	0.0	0.0
Penicillium 77-174	1.6	1.2	0.0	0.0	0.0	0.0
Trichoderma 77-119	3.0	1.2	2.7	1.5	1.7	2.5
Trichoderma 77-171	3.6	1.6	2.0	1.5	1.7	1.7
Bacterial sp. 78-10	1.3	1.2	1.7	1.1	1.5	1.1
Bacterial sp. 78-11	2.6	1.6	2.8	0.0	0.0	0.0
Bacillus 77-102	4.2	5.0	2.0	5.0	2.0	4.0
Bacillus 77-135	1.7	1.8	2.6	2.0	4.4	1.2
Bacillus 77-144	4.1	2.8	2.2	2.3	2.0	2.1
Pseudomonas 78-18	3.5	0.0	0.0	0.0	0.0	0.0
A. radiobacter W1	2.5	2.8	0.0	0.0	0.0	0.0
A. radiobacter 84	0.0	0.0	5.0	4.8	5.0	5.0

[a] Strains W1 and 84 were spotted at the center of mannitol-glutamate agar plates and grown for 3 days before being sprayed with suspensions of the test strains. All other antagonists were placed on the PDA plates in 7-mm diameter plugs from agar plate cultures immediately after the PDA plates were sprayed with the test strains.

TABLE 2. Effect of fungal and bacterial antagonists on formation of galls on tomato seedlings by biotype 1 and biotype 2 strains of *Agrobacterium tumefaciens*

Antagonists	Viable inoculum (propagules per ml)	Galled seedlings (%)[a]	
		EU-8	B234
Bacterial sp. 78-11	4.0×10^7	100	0
Pseudomonas 78-18	1.6×10^8	40	40
Bacillus 77-102	3.0×10^8	67	0
Bacillus 77-135	1.2×10^9	27	0
A. radiobacter W1	1.7×10^9	100	100
A. radiobacter 84	1.8×10^9	100	0
Penicillium 77-104	9.4×10^6	13	0
Aspergillus 77-148	4.9×10^6	0	13
Inoculated control		100	100

[a] Mean of five replications of three tomato seedlings per pot. The concentration of strain EU-8 was 1.5×10^7 viable cells per milliliter, and B234 was 7.0×10^6 viable cells per milliliter. Control seedlings wounded and inoculated with antagonists alone or with distilled water were not galled.

Greenhouse experiments. Six of the 16 antagonists tested on tomato seedlings reduced or prevented galling by the biotype 2 strain B234 below that of the control (Table 2). Five of these antagonists also reduced the formation of galls by strain EU-8, a biotype 1 strain not controlled by *A. radiobacter* strain 84.

For some antagonists, there was a low correlation between inhibition of agrobacteria in vitro (Table 1) and biological control of crown gall in the initial tomato assay (Table 2). One *Pseudomonas* antagonist (78-18) prevented galling by strains B234 and EU-8, but neither of these strains was inhibited by the *Pseudomonas* strain in vitro. More commonly, antagonists, such as *A. radiobacter* W1, failed to prevent galling by pathogenic strains that were sensitive in vitro.

Seven of the 16 antagonists also were tested against two mixtures of pathogenic strains to be used in field trials (Table 3). Both groups of pathogens contained agrocin 84-insensitive strains, and as expected strain 84 failed to prevent galling when coinoculated with these pathogens. However, strain 84 gave complete protection against all pathogens when inoculated 24 hr before the pathogens (Table 3). Of the new antagonists tested in this study, only the *Penicillium* and *Aspergillus* strains gave complete control of galling caused on tomato by both pathogen groups. Complete control by the *Penicillium* isolate occurred only when inoculation with the pathogen was delayed 24 hr; in contrast, *Aspergillus* controlled crown gall only if antagonist suspensions were mixed directly with those of the pathogens (Table 3).

TABLE 3. Effect of bacterial and fungal antagonists on the galling of tomato seedlings by two mixtures of *Agrobacterium* pathogens

Antagonists	Viable inoculum (propagules per ml)	Galling (%)[a] Group I pathogens		Group II pathogens	
		Coinoculated	24 hr[b]	Coinoculated	24 hr
Bacterial sp. 78-11	3.6×10^7	100	100	100	100
Bacillus 77-102	7.2×10^7	100	56	100	71
Bacillus 77-135	6.3×10^8	100	100	67	100
A. radiobacter W1	3.6×10^8	11	89	89	100
Pseudomonas 78-18	2.6×10^8	100	56	100	78
A. radiobacter 84	1.8×10^8	100	0	100	0
Penicillium 77-104	9.0×10^6	11	0	56	0
Aspergillus 77-148	8.5×10^7	0	100	0	100
Inoculated control		100	100	100	100

[a] Mean of three replications of three tomato seedlings per pot. Group I pathogens included strains A49, A432, A20, and A329 of *A. tumefaciens* at 2.9×10^7, 4.4×10^7, 1.1×10^8, and 3.3×10^7 viable cells per milliliter of inoculum, respectively. Group II included strains U-3, B6, B234, and EU-8 at 5.0×10^7, 1.9×10^6, 2.5×10^8, and 1.0×10^8 viable cells per milliliter of inoculum. Control seedlings wounded and inoculated with antagonists alone or with distilled water were not galled.

[b] Inoculations with the pathogens were 24 hr after inoculations with the antagonists.

Field tests for biological control. Most of the new antagonists were effective in reducing tomato seedling galling by both groups of pathogens when the pathogens were applied 24 hr after the antagonists (Table 4). Biological control was enhanced by the 24-hr delay for both fungal antagonists and for some of the bacterial antagonists. In addition, the *Penicillium* antagonist reduced galling by group II pathogens to a level below that of the wounded uninoculated controls, whether or not pathogen inoculation was delayed 24 hr. However, none of the antagonists isolated in this study were as effective as strain 84 for overall reduction of the galling of tomato seedlings by both pathogen groups. There was no observable phytotoxicity from any of the antagonists; treated plants were as healthy as untreated controls.

DISCUSSION

Biological control of crown gall was achieved by using fungal and bacterial antagonists other than *Agrobacterium radiobacter* strain 84. Previously strain 84 was the only antagonist reported to control crown gall, although many *Agrobacterium* strains have been tested (5,10,13). The level of control achieved with some of our new antagonists was comparable to that reported initially by Kerr (7) using strain 84. In his first report, Kerr found that galling was reduced from 79% to 31% when peach seeds were treated with strain 84. Results from the present study showed that the incidence of crown gall of cherry seedlings could be reduced from 61.9% to 4.1% by using a *Penicillium* antagonist against group II pathogens. The same antagonist reduced galling of tomato seedlings by group I pathogens from 81.4% to 32.8%. In addition, the mean level of naturally occurring infection (wounded uninoculated control) was reduced from 9.1% to 0.0% by both the *Penicillium* antagonist and *Bacillus* 77-135. The reduction of naturally occurring infection is one of the most important considerations, because the number of cells in *A. tumefaciens* culture inoculum far exceeds the natural level. Some biological control was achieved by the remaining antagonists, but not always with both groups of pathogens.

Biological control was enhanced when some of the antagonists were inoculated into wounded plant tissues 24 hr before the pathogens. The 24-hr delay may have helped the antagonists become established at the wound site, resulting in greater antibiotic production or better physical competition against the pathogen for infection sites. It is also possible that the antagonists stimulated production of substances by the host that inhibited the pathogens. In field tests, the 24-hr delay simply may have allowed more propagules of the antagonists to adsorb onto the cherry seedling roots, so they were not washed off in the subsequent pathogen dip. If true, the method of coinoculating antagonists and pathogens in

TABLE 4. Effectiveness of bacterial and fungal antagonists on crown gall of mazzard cherry seedlings in the field

Treatment	Viable inoculum (propagules per ml)	Galling (%)[a] Antagonist alone	Group I pathogens Immediate[b]	Group I pathogens 24 hr[c]	Group II pathogens Immediate	Group II pathogens 24 hr
Bacterial sp. 78-11	3.0×10^6	7.6 x	63.2	71.9	24.6	30.8
Pseudomonas 78-18	3.5×10^7	3.7 x	69.0 x	23.7	44.2 x	10.2
Bacillus 77-102	1.3×10^6	2.7	65.9 x	38.1	34.0	19.4
Bacillus 77-135	1.2×10^6	0.0	57.1	28.3	36.7	70.6 y
A. radiobacter W1	3.3×10^6	12.0 x	55.5	53.0	35.6	11.2
A. radiobacter 84	9.0×10^5	1.1	7.6	14.9	4.4	0.0
Penicillium 77-104	5.3×10^6	0.0	62.5	32.8	4.1	8.7
Aspergillus 77-148	4.0×10^6	4.1 x	80.8x	52.2	17.6	21.1
Inoculated control			81.4 x	...[d]	61.9 xy	...[d]
Wounded control		9.1 x				

[a] Mean of 100 cherry seedlings per treatment. Group I pathogens included strains A49, A432, A20, and A329 of *A. tumefaciens*. Group II pathogens included strains U-3, B6, B234, and EU-8. The concentration of the group I inoculum mixture was 5×10^4 viable cells per milliliter, and the group II mixture was 4.7×10^5 viable cells per milliliter. Means in a column followed by the same letter do not differ significantly ($P = 0.01$), according to the Z test.

[b] Seedlings were dipped in suspensions of individual antagonists 10 min before being dipped in pathogen mixtures.

[c] Seedlings were dipped in suspensions of individual antagonists 24 hr before being dipped in pathogen mixtures.

[d] ... Not tested (see discussion).

the field may have favored the pathogens. Wound healing following a 24-hr delay between application of the antagonists and pathogens probably did not enhance control; Moore (12) showed that 85% of mazzard cherry seedlings remained susceptible to infection for 3 days after wounding; Braun (2) also showed that *Kalanchöe* tissues were more susceptible to infection 24 hr after infection. There were also instances in greenhouse and field tests in which the 24-hr delay adversely affected control by some antagonists. Inoculum survival and colonization may have been poor during the 24-hr period for the *Aspergillus* antagonist on the aerial tomato stem, and for the bacterial strain 78-11 on cherry seedlings.

There was a low correlation between in vitro activity and biological control of some of the antagonists. *A. radiobacter* W1 inhibited *A. tumefaciens* EU-8 in vitro, but was ineffective in biological control on tomato. Kerr and Panagopolous (10) also isolated several bacteriocin-producing *A. radiobacter* strains that failed to control sensitive pathogenic strains on tomato. The failure to control sensitive pathogenic strains may result from a lack of bacteriocin production by the antagonists in vivo, mechanisms of biological control other than bacteriocin production, or poor growth of the antagonists at the wound site (4). On some hosts, *A. radiobacter* 84 has been shown to control pathogenic strains that were insensitive to agrocin 84 in vitro (13,17). This may suggest that other mechanisms, such as competition for infection sites (11), are utilized by strain 84 to prevent infection of some host species. In our work, strain 84 also prevented infection by insensitive strains on cherry seedlings, and on tomato seedlings inoculated 24 hr before the pathogens. However, recently we found that these "insensitive" pathogens are inhibited by strain 84 in vitro when grown on PDA, or on a minimal medium (mannitol-glutamate) to which glucose was added (D. Cooksey and L. W. Moore, *unpublished*). That the effect of glucose was on the sensitivity of the pathogen rather than on the production of agrocin by strain 84 was demonstrated by adding glucose to an overlay containing the test strain after strain 84 had been grown on the basal MG medium and killed with chloroform. Since sensitivity of these "insensitive" pathogens to strain 84 can be demonstrated in vitro using a specific growth medium, bacteriocin production is still a probable mechanism by which strain 84 controls these pathogens. The effect of different media on sensitivity of pathogens to strain 84 may also explain why the group I pathogens were controlled on cherry and pear, but not on apple seedlings (14) or tomato seedlings. The sensitivity of *A. tumefaciens* to agrocin 84 may be influenced by root exudates or other factors that differ between hosts.

The results of this investigation show that fungi and bacteria other than *A. radiobacter* strain 84 can be used as biological control agents for crown gall. The data also support the conclusion of Deep and Young (3) that fungal competitors play an important role in the natural incidence of crown gall. The *Penicillium* antagonist provided protection from naturally occurring *A. tumefaciens* strains throughout the growing season, and the initial inoculum level was comparable to that found naturally in soil from one Washington nursery. The possibililty of recolonizing fumigated nursery soils with selected antagonists also is being investigated.

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